Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Moda F, Gambetti P, Notari S, et al. Prions in the urine of patients with variant Creutzfeldt–Jakob disease. N Engl J Med 2014;371:530-9. DOI: 10.1056/NEJMoa1404401

Supplementary Materials

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Supplementary Methods

***PRNP* genotyping**

Urinary DNA was extracted from urine using the QIAamp DNA Micro kit (QIAGEN) with some modifications to the protocol. Three µl of urinary DNA were mixed with 15 µl of *Thermusacquaticus* polymerase (Taq 2X, New England Biolabs), 1 µl (100 ng/µl) of forward primer (GCAGTCATTATGGCGAACCTTG, Integrated DNA technology), 1 µl (100 ng/µl) of reverse primer (CCTTCCTCATCCCACTATCAGG, Integrated DNA technology) and 12 µl of nuclease free water. Polymerase chain reaction was performed (35 cycles) and the samples were loaded onto 1.5% agarose gel. Electrophoresis was carried out and the gel was soaked in gel red solution (Life technologies) for 1h, with shaking. Results were visualized under UV light (ChemiDoc XSR system) and the DNA fragment of interest was cut from the gel with a clean scalpel. Following the instruction provided with the QIAquick Spin Kit (QIAGEN), DNA was recovered from agarose gel, eluted in 30 µl of elution buffer and prepared for enzymatic digestion. Either Nsp1 (New England Biolabs) or HpyCH4IV (New England Biolabs) restriction

enzymes were used. The final reaction solutions (containing 1µl of restriction enzyme, 17 µl of PCR amplified fragments from urinary DNA and 2 µl of buffer 10X) were incubated 2 h at 37°C and then samples were separated by electrophoresis on 1.5% agarose gel. The gel was soaked in gel red solution for 1 h (with shaking) and the results were visualized under UV light.

Supplementary Figures

Figure S1. **Lack of PrP^{Sc} detection in urine by Western blot and optimization of PMCA using urine samples supplemented with variant Creutzfeldt-Jakob disease brain homogenate. A**: As a first attempt to detect PrP^{Sc} in urine we tried direct Western blot after PrP^{Sc} concentration by high speed centrifugation. PrP^{Sc} from 1 ml of urine was precipitated by centrifugation at 100,000 x g (1h, 4°C). After washing, the resulting pellets were resuspended in 10 µl of PBS (100-fold concentration). Samples were digested with PK (100 µg/ml, 1h, 37°C) and after blotting were analyzed with the 3F4 monoclonal antibody. As controls, we used a 10% brain homogenate of a vCJD patient and a 10% normal brain homogenate (NBH) of mice expressing human PrP. This picture corresponds to a representative figure of all the samples analyzed. No PK-resistant PrP^{Sc} signal was observed in any of the urine samples studied. **B**: To evaluate the possibility of cross-contamination or de novo generation of PrP^{Sc} after PMCA, healthy urine supplemented with 10% brain homogenate from an individual not affected by prion disease was processed in the same manner as the samples analyzed in figure 2B and subjected to 7 serial rounds of PMCA in the presence of 10% transgenic mice brain homogenate as substrate.

As positive controls, the same experiments were simultaneously done in the presence of 10% vCJD brain homogenate. NBH: normal transgenic mice brain homogenate; Mk: molecular weight marker; HC: healthy control; PD: Parkinson disease; AD: Alzheimer's disease; FTD: fronto-temporal dementia. All samples were treated with PK, except the NBH, used as a migration control for PrP^{C} electrophoretical mobility

Figure S2. Estimation of PrP^{Sc} concentration in urine by quantitative PMCA. **A**: PrP^{Sc} concentration in a vCJD brain homogenate was estimated by comparing the Western blot signal of various dilutions of vCJD brain homogenate treated by PNGase F and PK (100 µg/ml) with that of known quantities of recombinant PrP. This procedure enabled to estimate that PrP^{Sc} concentration in the brain of this vCJD patient was approximately 100 µg/g. **B**: Healthy urine samples were supplemented with known concentrations of vCJD PrP^{Sc}. Samples were processed as described in Methods (figure 1) and subjected to various rounds of 96 PMCA cycles. PrP^{Sc} signal was detected by Western blot after PK digestion. All samples were treated with PK, except the NBH used as a PrP^{C} migration control.

FIGURE S1

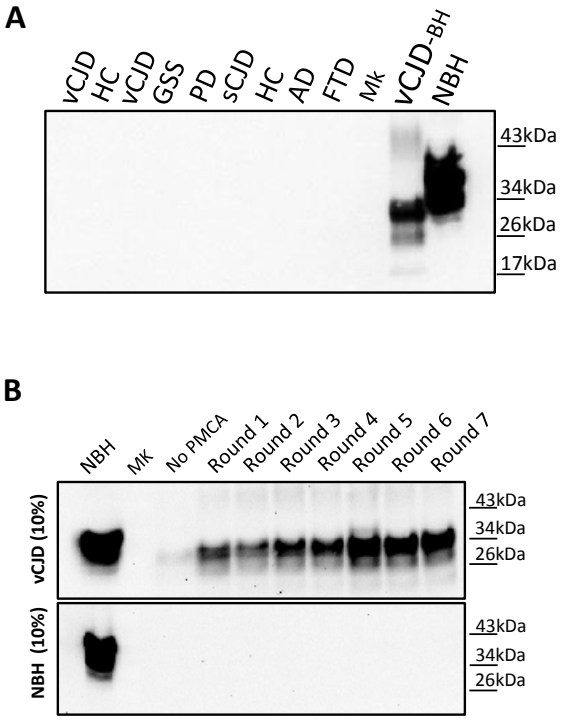
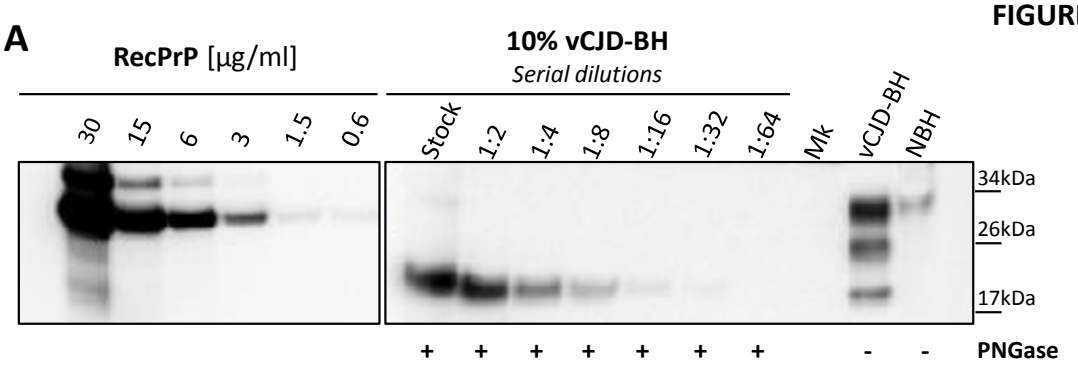

A
vCJD
HC
vCJD
GSS
PD
sCJD
HC
AD
FTD
Mk
vCJD-BH
NBH
43kDa
34kDa
26kDa
17kDa
B
NBH
MK
No PMCA
Round 1
Round 2
Round 3
Round 4
Round 5
Round 6
Round 7
vCJD (10%)
NBH (10%)
43kDa
34kDa
26kDa
43kDa
34kDa
26kDa

FIGURE S2


A
RecPrP [µg/ml]
30
15
6
3
1.5
0.6
10% vCJD-BH
Serial dilutions
Stock
1:2
1:4
1:8
1:16
1:32
1:64
Mk
vCJD-BH
NBH
34kDa
26kDa
17kDa
+ + + + + + +
- -
PNGase

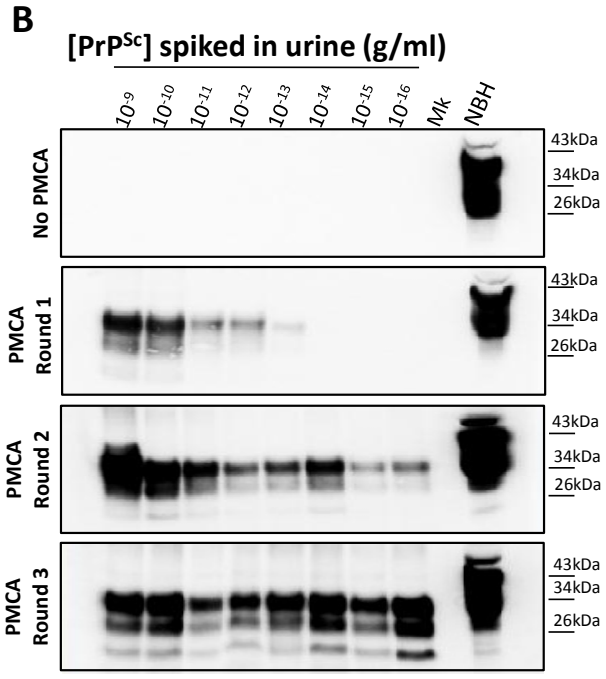

B
[PrPSc] spiked in urine (g/ml)
10-9
10-10
10-11
10-12
10-13
10-14
10-15
10-16
Mk
NBH
No PMCA
PMCA Round 1
PMCA Round 2
PMCA Round 3
43kDa
34kDa
26kDa

TABLE S1. Clinical information for the vCJD patients studied.

Case	Age at onset	Disease duration (months)	Treatment
vCJD, patient1	22	32	None
vCJD, patient 2	27	26	Doxycycline
vCJD, patient 3	20	27	None
vCJD, patient 4	25	27	None
vCJD, patient 5	28	17	None
vCJD, patient 6	33	19	None
vCJD, patient 7	31	13	None
vCJD, patient 8	17	114	PPS
vCJD, patient 9	28	11	None
vCJD, patient 10	39	16	PPS
vCJD, patient 11	32	17	None
vCJD, patient 12	28	23	None
vCJD, patient 13	18	11	None
vCJD, patient 14	21	30	None